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Sensitivity, polarity, and conductance change in the response of vertebrate hair cells to controlled mechanical stimuli

(sensory transduction/intracellular recording/amphibian/sacculus/action potential)

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ABSTRACT **Hair cells, the primary receptors of the auditory, vestibular, and lateral-line sensory systems, produce electrical signals in response to mechanical stimulation of their apical hair bundles. We employed an *in vitro* preparation and intracellular recording to investigate the transduction mechanism of hair cells in the sacculus from the inner ear of the bullfrog (*Rana catesbeiana*). When stimulated directly by mechanical deflection of their hair bundles, these cells gave graded responses up to 15 mV in amplitude; the peak sensitivity was about 20 mV/μm deflection. The depolarizing component of the receptor potential corresponded to stimuli directed towards the kinocilium. Depolarizing responses were associated with a membrane resistance decrease, and hyperpolarizing responses with a resistance increase. Action potentials, possibly calcium spikes, were occasionally evoked in hair cells by mechanical or electrical stimulation.**

Vertebrates detect sounds, body accelerations, and water movements with the acoustico-lateralis sensory system. The primary receptors of this system, whether in the cochlea, the vestibule, or the lateral-line organ, are neuroepithelial cells termed hair cells. These cells are characterized by the presence at their apical surfaces of "hair" bundles, each consisting of elongated microvilli (stereocilia) and in most cases a single true cilium (kinocilium). Stimuli of various modalities, conveyed to the appropriate hair cells in the form of vibrations or static deformations, stimulate the cells by bending their hair bundles. Hair cells respond with small receptor potentials (1, 2) which evidently excite afferent nerve fibers by chemical (3) or electrical synapses.

The transduction process by which bending of the hair bundle elicits an electrical response is poorly understood because hair cells are generally small, relatively inaccessible, and difficult to impale with intracellular microelectrodes. Moreover, because the cells *in situ* are stimulated through the mechanical and hydrodynamic linkages that couple their hair bundles to vibrations in the external medium, the exact nature of the mechanical stimuli delivered to the cells is unknown in every instance. We have circumvented these difficulties by developing an *in vitro* preparation which permits intracellular recording from relatively large hair cells during the delivery of precisely defined stimuli directly to their hair bundles.

MATERIALS AND METHODS

Experimental Preparation. The hair cell preparation was taken from the sacculus of the bullfrog (*Rana catesbeiana*); this organ of the inner ear responds to ground-borne vibrations (4) and perhaps to sound (5). The macula, a discoidal region of the saccular wall in which the hair cells are situated (6), was rapidly dissected from the labyrinth by a ventral surgical approach. After the otoconia (otoliths) within the sacculus were rinsed away with a gentle stream of standard saline solution, the transparent otolithic membrane that overlies the macula was carefully peeled away with fine forceps. The preparation, roughly 1 mm in diameter, was then placed, hair cells uppermost, upon a cover slip at the bottom of an 0.5-ml experimental chamber and secured by tungsten spring clips. The chamber was placed on the fixed stage of a modified microscope (model WL, Carl Zeiss, New York, NY) and observed during an experiment with Nomarski differential interference contrast optics through a 40×, water-immersion lens of numerical aperture 0.75.

Throughout an experiment, the preparation was superfused at a rate of about 1.5 ml/min with saline solution. The standard medium contained 113 mM Na^+, 2 mM K^+, 4 mM Ca^{2+}, 123 mM Cl^-, and 3 mM D-glucose, buffered to pH 7.3 with 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes). Magnesium saline was identical except that 4 mM Mg^{2+} replaced the Ca^{2+}. Tetrodotoxin and streptomycin salines contained, respectively, 10 μM tetrodotoxin (Calbiochem, San Diego, CA) and 100 μM streptomycin sesquisulfate (Sigma, St. Louis, MO) added to the standard medium. The experimental chamber and all solutions were maintained at 20–22°, and the preparation was protected from radiant heating with an infrared interference filter in the microscope illumination system.

Recording Apparatus. The limited working distance of the objective lens and the fragility of the hair cells necessitated the development of mechanically stable, fine-tipped glass microelectrodes bent near their tips to allow vertical penetrations of cells with horizontally positioned electrodes. Bending was accomplished by placing the distal 200 μm of each electrode into a drop of distilled water and applying heat from a platinum filament just outside the meniscus (A. J. Hudspeth and D. P. Corey, unpublished data). The water droplet protected the electrode tips from partial melting. By virtue of its surface tension, the water also controlled the angle of bending: the electrodes bent until they were perpendicular to the air–water interface at the point of entry. Electrodes were then stiffened mechanically by cementing a solid, 200-μm-diameter glass strut between the bend point and the shank. Most electrodes were pulled from capillaries containing a fiber of the same glass, and filled by capillarity with 3 M KCl. Tip resistances were 300–500 MΩ in standard saline solution.

Intracellular potentials were recorded with a high-impedance preamplifier (model 701, W-P Instruments, Hamden, CT), amplified (model AM 502, Tektronix, Beaverton, OR), and stored on magnetic tape for subsequent analysis. The instrumentation recorder (model 3960, Hewlett-Packard, San Diego, CA) provided a frequency response from dc to 5 kHz with FM recording. To minimize an electrode noise level of roughly 1 mV, responses were usually averaged with a digital signal averager (model 4620/4623, Ortec, Oak Ridge, TN). Intracellular current pulses were generated by a stimulator (model 4610 system, Ortec) and passed through the recording electrode; a "bridge" circuit in the preamplifier permitted measurements

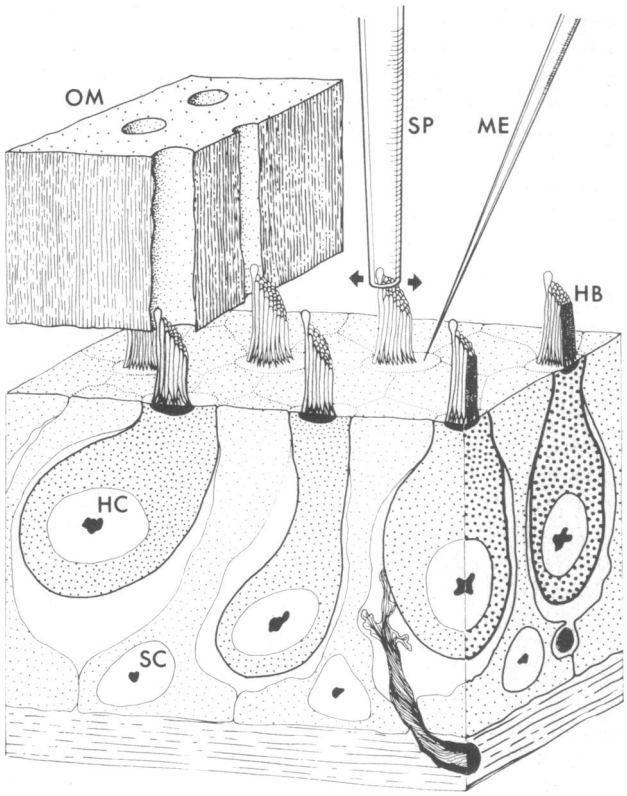

FIG. 1. Schematic cutaway of the experimental preparation. Hair cells (HC) and supporting cells (SC) form an epithelial sheet supported by a layer of connective tissue. The otolithic membrane (OM), which normally couples stimuli to the hair bundles (HB) of the receptor cells, is shown partially dissected from the site of experimentation. While the intracellular potential is recorded through a glass microelectrode (ME), a hair cell is stimulated by a capillary stimulus probe (SP) slipped over the tip of its hair bundle and moved parallel to the epithelial surface (arrows).

of intracellular potential during current injection. The current delivered was monitored by a virtual ground circuit interposed between the silver/silver chloride bath electrode and ground.

Stimulating Apparatus. Mechanical stimuli were conveyed to individual hair cells by glass capillary probes slipped over the tips of their hair bundles. Capillaries were pulled on conventional microelectrode pullers and bent as described above. Under a dissection microscope, they were ground flat to internal tip diameters of 2–3 μm with 0.3-μm-diameter abrasive particles on plastic discs (Imperial Lapping Film, Minnesota Mining and Manufacturing, St. Paul, MN) mounted on a rotary grinding tool.

Probes were attached to and actuated by a piezoelectric bimorph bender element (Vernitron, Bedford, OH) driven by a waveform generator (model FG 501, Tektronix). Like the recording electrodes, the stimulus probes were about 30 mm in length, and were positioned with a micromanipulator (Leitz, Wetzlar, Germany). The motion of the stimulus probe was calibrated with the experimental microscope and stroboscopic illumination to an accuracy of about ± 0.1 μm. In the frequency range from dc to 150 Hz, this system displayed good linearity, uniform frequency response, negligible hysteresis, and no resonances. The probe tip moved along a virtually straight line parallel to the experimental preparation's surface.

Histology. Living experimental preparations were photographed through Nomarski optics. The lengths of hair bundles were estimated both by viewing sacculi tangentially to their apical surfaces and by using the calibrated fine focusing adjustment to measure the distances of hair bundle tips from apical surfaces.

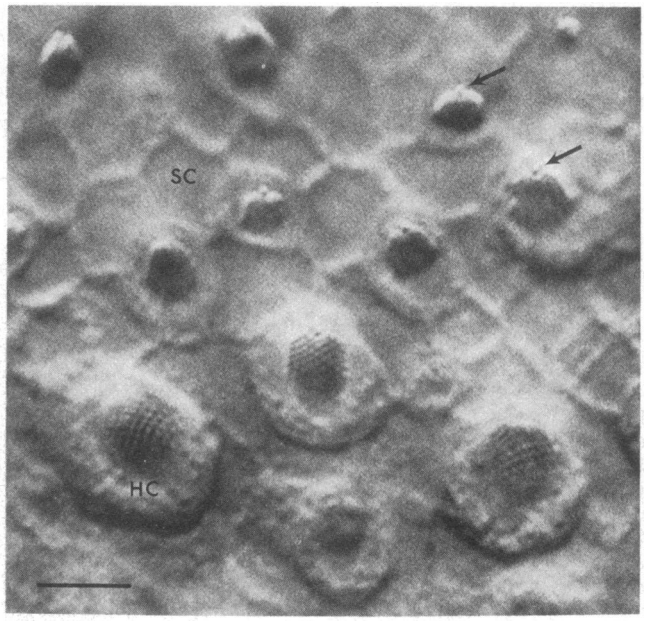

FIG. 2. Differential interference contrast photomicrograph of a living bullfrog saccular preparation. This view shows the rounded apical surfaces of several hair cells (HC) and the polygonal surfaces of the supporting cells (SC) which separate them. The single kinocilia of some hair cells are apparent (for example, at arrows); individual stereocilia can be observed in the hair bundles in the lower portion of the figure. Note the common orientation from cell to cell of the morphological vector running from the clustered stereocilia to the kinocilium. (×1200; scale bar = 10 μm.)

Sacculi to be sectioned were fixed *in situ* by irrigation with a fixative containing 200 mM glutaraldehyde and 5 mM $CaCl_2$ buffered to pH 7.3 with 80 mM sodium cacodylate. After dehydration in ethanol, specimens were embedded in epoxy plastic. Sections cut on glass knives at a thickness of 1 μm were stained with toluidine blue.

RESULTS

Anatomy

The macular region of the bullfrog sacculus consists of a sensory epithelium overlying a thick sheet of connective tissue. The epithelium from a mature animal contains approximately 3000 hair cells, of which the majority are roughly 25 μm long and 15 μm in greatest diameter (Figs. 1 and 2). The hair cells are generally separated from one another by columnar supporting (sustentacular) cells, to which they are attached at their apical borders. The apical surface of each large hair cell is round or slightly ovoid, about 7 μm in diameter, and endowed with an eccentrically placed hair bundle comprising a single kinocilium and roughly 50 stereocilia. Afferent and efferent nerve fibers contact the hair cells on their basal and lateral aspects.

The apical boundaries of hair and supporting cells are readily apparent in living preparations under the Nomarski viewing system (Fig. 2). The kinocilium and individual stereocilia of each cell are also usually visible, so that one may define a vector, from the center of the stereociliary mass to the kinocilium, which corresponds to the axis of greatest sensitivity of the cell (7). The orientations of hair cells shift systematically across the macular surface, and reverse along a curved boundary (6).

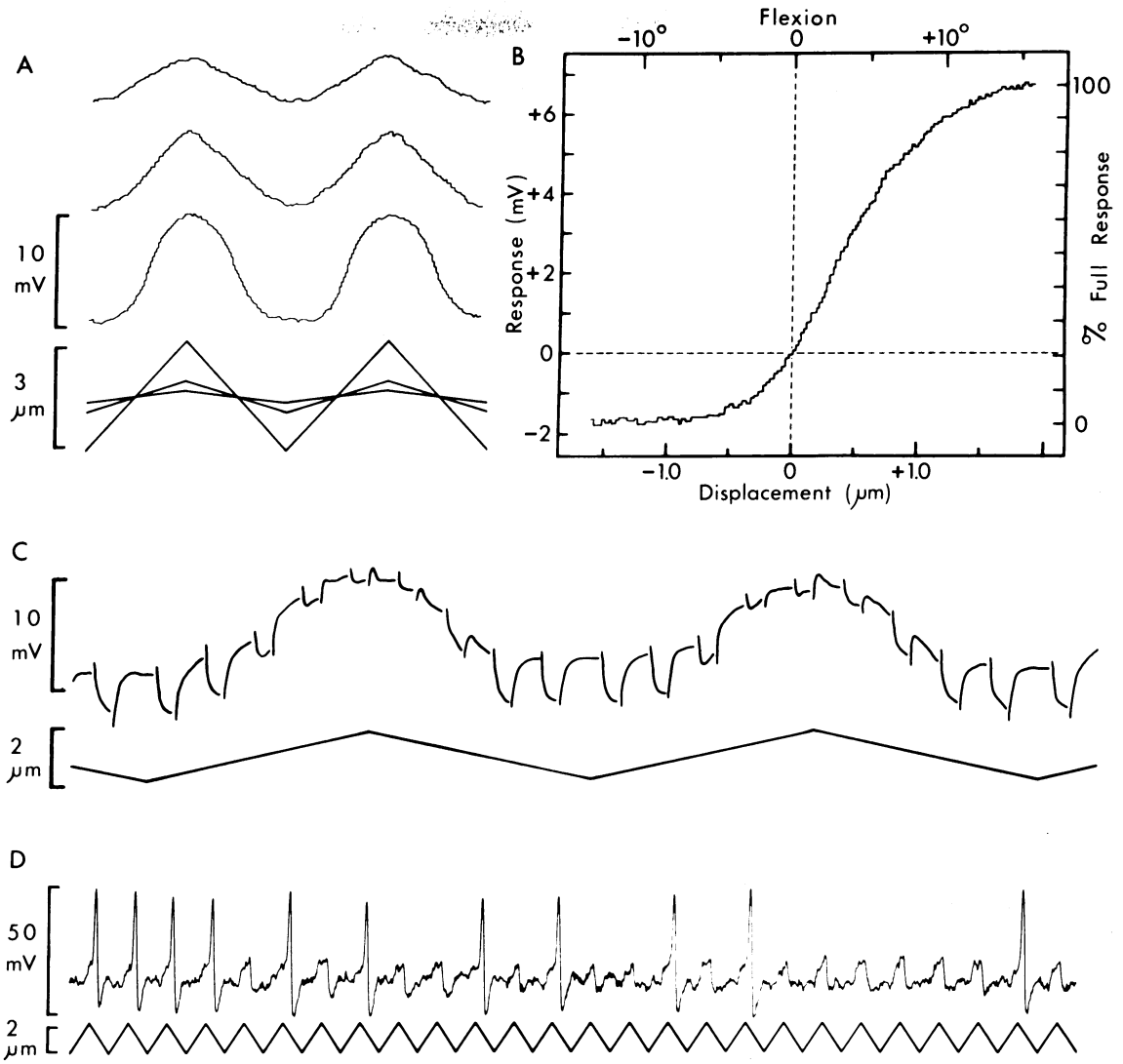

FIG. 3. (*A*) Receptor potentials recorded from a bullfrog saccular hair cell during direct stimulation of its hair bundle. The stimulus probe was driven with 10-Hz triangle waves of three amplitudes (superimposed lowest traces) while the intracellular potential was measured with a microelectrode; each record (upper three traces) is the average of 32 responses. The response to low-amplitude stimulation follows the driving signal (top trace), whereas higher amplitudes elicit markedly clipped responses (second and third traces). (*B*) The input-output relationship for the hair cell whose responses are depicted in *A*. The curve shows the potential change from the resting potential (−58 mV) as a function of the displacement of the hair bundle's tip by a 10-Hz triangle wave stimulus. The zero point, which was measured with the stimulating probe removed, is accurate to only ±1 mV because of the electrical noise level. An alternate abscissa represents the estimated angle of flexion of the 9-μm-long hair bundle on the assumption that it pivots at its base. The input-output curve is markedly asymmetrical: the positive response evoked by movements towards the kinocilium exhibits a more gradual approach to plateau and reaches a greater absolute magnitude than the negative response on movement towards the stereocilia. (*C*) Changes in the input resistance of a hair cell during its response to mechanical stimulation. Hyperpolarizing square current pulses (70 pA, 45 msec) were injected into a cell while its hair bundle was deflected at 1 Hz (lower trace); the bridge circuit used in recording was balanced for the depolarizing phase of the response. The potential change produced by the current pulses, an index of cell input resistance, is superimposed upon the receptor potential (upper trace). Sixteen responses were averaged for this figure. The record demonstrates a decrease in the membrane resistance during depolarizing phases of the response and an increase for hyperpolarizing responses. (*D*) Action potentials arising from the depolarizing phases of receptor potentials in a saccular hair cell. The cell, which had a resting potential of −65 mV, was stimulated with a 10-Hz triangle wave motion (lower trace) applied to its hair bundle. Note that the action potential occasionally fails, leaving a pure receptor potential.

Intracellular responses

Most recordings involved relatively large hair cells at the macular periphery opposite the origin of the saccular nerve. Cells were generally impaled through their apical surfaces, opposite the kinocilia; similar results were obtained from penetrations of the perikaryal region. Although highly dependent on the quality of electrodes and mechanical stability, resting potentials were usually 50–60 mV and occasionally up to 70 mV, inside negative. The present results, derived from 23 animals, are based upon records from 73 cells with resting potentials of 50 mV or greater. All of these cells were held for at least 5 min, and a few for over 60 min, without significant changes in their resting potentials.

Stimuli applied to hair bundles elicited graded potentials usually 5–10 mV, but occasionally more than 15 mV, in amplitude (Fig. 3*A*). The cells responded to static deflections and to sinusoidal stimuli of frequency up to at least 150 Hz. Within this frequency range, the output of the hair cell transduction apparatus depended on hair bundle displacement, but not on velocity. In each of 61 cells for which the morphological polarity of the hair bundle was evident, the depolarizing phase of the

response occurred on bending of the hair bundle towards the kinocilium, and the hyperpolarizing component on movement towards the stereocilia. Pushing downwards on the hair bundle by lowering the probe also provoked depolarization of amplitudes similar to those of responses to bending.

The input–output relationship for hair cells was determined with good agreement from responses either to periodic signals or to static displacements. While the absolute magnitudes of responses vary, the shapes of normalized input–output curves (Fig. 3*B*) are similar from cell to cell. The position of zero hair bundle deflection was determined by removing and replacing the stimulating probe during the course of an experiment. At low stimulus amplitudes, corresponding to hair bundle tip displacements of up to about ± 0.4 μm, the response is roughly linear; at higher amplitudes there is pronounced saturation of the response at both extremes of flexion. The input–output curve is accordingly sigmoidal, but asymmetrical; the response saturates more abruptly on bending of the hair bundle towards the cluster of stereocilia than on flexion towards the kinocilium.

Several lines of evidence indicate that the responses we recorded were receptor potentials, rather than artifacts of cell or electrode movement induced by mechanical stimulation. No typical responses were observed until the vibrating probe contacted the hair bundle; only small and asynchronous signals resulted from application of the stimulating probe directly to the apex of the hair cell or to the recording electrode. The polarity of the response was constant regardless of the geometry of the stimulating and recording electrodes, and reversed at the anatomical boundary in the macula at which hair cell polarities reverse. Replacement of Ca^{2+} with Mg^{2+}, which diminishes extracellularly recorded hair cell responses (8), reversibly reduced the intracellular response. Finally, 100 μM streptomycin, an aminoglycoside drug that interferes with hair cell transduction (9), rapidly and reversibly blocked the response without abolishing the resting potential.

Conductance changes

The receptor potentials of other primary sensory receptors are caused by changes in transmembrane ionic current consequent upon stimulus-induced alterations in specific ionic conductances (10–13). If the vertebrate hair cell response has a similar origin (14, 15), it should be possible to detect the conductance change involved. For this purpose, responses to stimuli of saturating intensity were collected while short, constant-current pulses were injected into cells through the recording electrode. The potential changes produced by these pulses then provided an instantaneous measure of membrane conductance.

It has been possible to measure conductance changes in seven of roughly twice as many attempts; positive results are extemely dependent on optimal electrode penetrations. The resting input resistances of saccular hair cells are at least 200 MΩ; bending of hair bundles towards kinocilia decreases this resistance, while bending towards stereocilia increases it (Fig. 3*C*). The total resistance change between the extremes of response reached 50 MΩ; control experiments indicate that this change cannot be accounted for by the passive rectifying properties of the hair cell membrane.

Action potentials

A surprising feature of recordings from hair cells of the bullfrog sacculus was the occasional presence of action potentials. These were relatively slow, with risetimes of 5–10 msec, and their peak amplitudes rarely exceeded 40 mV above the resting potential (Fig. 3*D*). The largest action potentials also had undershoots of more than 15 mV. Impulses were observed only with optimal electrode penetrations, usually with resting potentials exceeding 60 mV. Single or multiple action potentials arose from the depolarizing phases of receptor potentials, or could be evoked by injection of positive current pulses. The spikes were not abolished or altered by the presence of 10 μM tetrodotoxin in the bathing medium.

DISCUSSION

The results presented here demonstrate that vertebrate hair cells can produce receptor potentials up to at least 15 mV in amplitude. This value approaches that found in invertebrate hair cells (12, 13) and in such other primary sensory receptors as photoreceptors (10) and invertebrate stretch receptors (11). The responses we have recorded are 4-fold larger than any noted previously (1–3), a fact we attribute to the exceptionally stable microelectrode penetrations afforded by special electrodes and an *in vitro* preparation. Because these cells seem to be quite sensitive to microelectrode penetration, and because their input resistances are very large and variable, it is likely that even the present electrodes compromised input resistances significantly and that still larger responses can be obtained.

In the inner ear, the apical surfaces of hair cells face a high-potassium, low-sodium endolymph solution, while the basal and lateral cell surfaces are in perilymph of an ionic composition similar to that of plasma. In the *in vitro* system described here, however, all surfaces of the cells are immersed in a fluid of homogeneous composition. It is therefore possible that this ionic irregularity, or the slightly elevated Ca^{2+} concentration (4 mM), is responsible for the large responses observed.

The form of the input–output relationships recorded intracellularly from single hair cells resembles the relationship found previously from extracellular (microphonic) recordings of large populations of cells. In particular, the asymmetry of the curve, with more rapid saturation in the hyperpolarizing than in the depolarizing direction, accords with the finding in the goldfish sacculus (16). The polarity of the response, which is positive on flexion towards the kinocilium, is consistent with inferences originally made from responses of the semicircular canal ampulla (7). The range of hair bundle displacements to which the cells respond is also comparable with values from other acoustico-lateralis organs. The normal range of displacement in the human semicircular canal is estimated on theoretical grounds not to exceed 3 μm (17). In the skate semicircular canal, recordings of nerve activity indicate an upper limit for the dynamic range of 5 μm, which corresponds to about 7° (18).

The sensitivity of the hair cells is extraordinary: the slope of the input–output curve can reach 20 mV per micrometer of displacement. If hair cells, like photoreceptors, can synaptically transmit statistically significant signals corresponding to 10 μV receptor potentials (19), the threshold sensitivity of the amphibian sacculus would approximate 500 pm (5 Å). Von Békésy estimated the movement of the basilar membrane of the human cochlea (approximately equal to bundle displacement) at 1 pm at auditory threshold (20). Although discovery of nonlinearity in the membrane response (21) prompts upward revision of this value, the sensitivity of cochlear hair cells is evidently much greater than that of amphibian saccular hair cells. We should note, however, that cochlear hair cells lack kinocilia and so may operate in a manner different from most other hair cells (22).

The difficulty in obtaining good records of conductance changes associated with hair cell responses leads us to regard this result as preliminary. Nevertheless, the depolarizing portion of the responses was consistently accompanied by a decrease

in input resistance. This suggests that the receptor potential of the vertebrate hair cell results from alterations in membrane conductance to an ionic species whose reversal potential lies positive to the resting potential. This observation and the blocking effect of Mg^{2+} are consistent with a role of Ca^{2+} in the transduction process (8).

There are several possible origins for the action potentials we have recorded from hair cells. Spikes might arise in the hair cells themselves, which would be expected to possess the voltage-sensitive channels associated with synaptic release (23). Alternatively, an endogenous action potential mechanism could serve as an amplifier of receptor potentials, as probably occurs in the modified acoustico-lateralis cells of electroreceptor systems (24). Finally, the spikes could be a manifestation of electrical coupling of hair cells to postsynaptic nerve terminals. While freeze-fracture examination of the bullfrog sacculus indicates that gap junctions on hair cells are rare, small junctions have been seen on hair cells situated peripherally in the macula (A. J. Hudspeth, unpublished data). Because of the slow time scale of the spikes and their insensitivity to 10 μM tetrodotoxin, we suspect that they are calcium action potentials (25) endogenous to the hair cells. While it is possible that the impulses are an artifact of elevated Ca^{2+} concentration, and are not normally a component of the response, they indicate the presence of a voltage-sensitive conductance mechanism in hair cells.

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